

# High Molecular Weight Neurofilament Proteins Are Physiological Substrates of Adduction by the Lipid Peroxidation Product Hydroxynonenal\*

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Protein adducts of the lipid peroxidation product *trans*-4-hydroxy-2-nonenal (HNE) are features of oxidative damage in neuronal cell bodies in Alzheimer's disease but are also seen in axons of normal as well as diseased individuals. In this study, focusing on the axons of the mouse sciatic nerve, we found that HNE adducts characterize axons of mice from birth to senility. Immunoblots of axonal proteins showed that HNE adducts are only detected in neurofilament heavy subunit (NFH) and, to a lesser extent, neurofilament medium subunit (NFM), both lysine-rich proteins, consistent with the adducts being limited to lysine residues. *In vitro*, HNE treatment of permeabilized sciatic nerve showed the same specificity, i.e. NFH and NFM are the only proteins that reacted with HNE, providing they are phosphorylated. Quantitative immunoblot analysis of two strains of mice ages 1–33 months showed that the levels of HNE adducts on NFH are consistent throughout life. Additionally, mice transgenic for human superoxide dismutase-1 with G85R mutation show no difference in HNE adduction to NFH compared with controls. Taken together, these studies indicate that HNE adduction to NFH is physiological, and its constancy from birth to senility as well as its dependence on phosphorylation argues that NFH and NFM modification may play a role in protecting the membrane-rich axon from toxic aldehydes resulting from oxidative damage.

One of the most striking and earliest changes noted with the development of Alzheimer's disease (AD)<sup>1</sup> is that cell bodies of vulnerable neurons uniformly show increased oxidative damage. Nitration (1, 2), reactive carbonyls (3), nucleic acid oxida-

tion (4), and adduction by glycoxidative intermediates (5) as well as the reactive lipoxidation product, *trans*-4-hydroxy-2-nonenal (HNE) (6, 7), are prominent. Although these changes are globally noted, there have been few indications as to the specific proteins displaying damage in human brain. However, the finding that not only are glycoxidation and lipoxidation adducts increased in neuronal cell bodies of AD cases but such reactive aldehyde adducts were also present in axons in the white matter of infants and aged controls in addition to patients with AD (7, 8) led us to consider whether specific axonal proteins are physiological targets of oxidative modification. In this study, we decided to focus our analysis on HNE adducts because they are the most readily detected and best characterized of the aldehyde-derived adducts. HNE is highly reactive and considered one of the most neurotoxic aldehydes produced *in vivo* (6). In addition, whereas HNE adducts are among the best characterized chemically, it is likely that HNE can be used as a model for the other reactive aldehydes produced by lipoxidation or glycoxidation because they have common features. The prevalence of HNE adducts led us to ask whether HNE adduction is an age- or axon transport-dependent process because the rate of adduction of HNE to target molecules should be first order and increase in an age-dependent manner such that reversible (e.g. lysine-Michael) (9, 10) and stable adducts (lysine-based pyrrole) (11, 12) of HNE may show distinct differences during the aging process.

Because preliminary immunoblotting studies of the brain and peripheral nerve of human (control and AD), rat, rabbit, and mouse showed that the only proteins displaying readily detectable HNE adducts are neurofilament heavy subunit (NFH) and neurofilament medium subunit (NFM), we decided to focus the analysis on the axons of the sciatic nerve as a natural site rich in NFH. NFH and NFM are the major proteins of the axon, and their transport from the cell body into segments to the terminals can be followed by dividing the nerve into segments. The high susceptibility of NFH and NFM to HNE adduction was maintained *in vitro*, where HNE treatment of permeabilized axon or enriched neurofilament proteins also led to specific modification of NFH and NFM, with no other proteins displaying any similar high affinity for HNE adduction. HNE adduction is likely related to the molecular structure of NFH and NFM because the adducts were limited to lysine residues, which are abundant on both these proteins. Additional specificity for adduction is provided by the phosphate

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The abbreviations used are: AD, Alzheimer's disease; HNE, *trans*-4-hydroxy-2-nonenal; NFH, neurofilament heavy subunit; NFM, neurofilament medium subunit.

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residues, whose presence was essential for NFH susceptibility to HNE adduction. Surprisingly, analysis of HNE adduct levels on NFH, both reversible and stable, showed consistent levels of modification with passage along the axon during aging and conditions of oxidative stress. These findings suggest that the levels of HNE adducts are regulated throughout life.

## EXPERIMENTAL PROCEDURES

**Preparation of Sciatic Nerve.**—C57BL6J/NIA mice ( $n = 27$ ; 3 of each age (1, 2, 3, 6, 9, 12, 15, 21, and 33 months)) and B6C3F1 mice ( $n = 27$ ; 3 of each age (3, 6, 12, 15, 18, 21, 25, 30, and 33 months)) were obtained from the National Institute on Aging colony at Charles River and maintained at the Case Western Reserve University Animal Facility under an approved protocol for 7–10 days before sacrifice. Euthanasia was induced by an overdose of pentobarbital before dissection. Animals were cooled on ice immediately upon death and maintained on ice during dissection. Under a stereomicroscope (Zeiss), the entire sciatic nerve, beginning within the spinal column and extending to the soleus muscle, was exposed, dissected, and divided into three segments: (i) proximal, within the spinal column, (ii) medial, extending from the spinal column to the beginning of the femur, and (iii) distal, from the femur to the entry into the muscle.

Tissue was disrupted with a ground glass homogenizer in ice-cold 1% Triton X-100, 0.1 M Tris-HCl, pH 7.6, containing 1.0 mM phenylmethylsulfonyl fluoride, 5  $\mu$ M leupeptin, and 20 mM EDTA for 30 min. The homogenate was centrifuged at  $10,000 \times g$  for 15 min, and the Triton-soluble fraction was separated from the Triton-insoluble fractions. The pellet was resuspended in 0.1 M Tris-HCl, pH 7.6, 1% SDS containing 1.0 mM phenylmethylsulfonyl fluoride, 5  $\mu$ M leupeptin, and 10 mM EDTA and frozen in liquid nitrogen. Protein was assayed by using the BCA method (Pierce). After PAGE (13) of equal protein loads (30  $\mu$ g of protein per one-third of a minigel; Bio-Rad), proteins were electrotransferred (30 V, 16 h) to Immobilon (Millipore) (14). Blots were blocked in 10% dry milk for 1 h on a shaker and rinsed in Tris-buffered saline, and the primary antibody was applied and incubated for 16 h at 4 °C. After five washes of 5 min in Tris-buffered saline-Tween, peroxidase-labeled secondary antibodies were applied for 1 h at room temperature. After rinsing again as described before, the blots were developed together for the same length of time using 3,3'-diaminobenzidine as co-substrate. Immunoblots from the proximal, medial, and distal segments from each animal were stained in parallel and under identical conditions for each antibody.

Enriched neurofilament fractions were also prepared from the sciatic nerve by using established procedures (15).

**Immunocytochemistry of Tissue Sections.**—Samples (1–2 mm long) were taken from the same segments analyzed for protein and fixed in methacarn (methanol:chloroform:acetic acid, 60:30:10) by immersion at 4 °C for 16 h and then transferred to 70% ethanol. The pieces were then embedded in 10% Bacto agar to obtain the correct orientation for cross-sectioning, dehydrated in graded ethanol and xylene, and embedded in paraffin. Sections were cut 6- $\mu$ m thick and placed on silane-coated slides (Sigma). After rehydration, antigens were localized by use of the peroxidase-antiperoxidase technique (16) with 3,3'-diaminobenzidine as co-substrate.

**Antibodies.**—The antibodies used were: (i) HNE-Michael, rabbit antiserum to the products of HNE adduction to keyhole limpet hemocyanin involving mainly Michael adducts of cysteine, histidine, and lysine (17), (ii) HNE-pyrrole, rabbit antiserum to the 4-oxononanal-modified keyhole limpet hemocyanin, which is specific for a stable pyrrole resulting from dehydrative evolution of the HNE-lysine Schiff base adduct (12), (iii) HNE-lysine-lysine, two different rabbit antisera to a fluorescent product of HNE cross-linking two lysine residues (18, 19), (iv) mouse monoclonal antibody to phosphorylated NFH (SMI-34; Sternberger Monoclonals), (v) mouse monoclonal antibody to nonphosphorylated NFH (SMI-32; Sternberger Monoclonals), and (vi) mouse monoclonal antibody to NFM (BioMedia).

Specificity of the various antibodies was verified in all cases by immunoprecipitation for 16 h at 4 °C with the competing antigens or to adducts prepared from the reaction of HNE with lysine, cysteine, or histidine at a 1:1 concentration of 0.8 mM. Reaction of the latter two amino acids with HNE gives the corresponding Michael adduct nearly stoichiometrically, whereas the reaction of HNE with lysine leads to a time-dependent mixture of mainly the reversibly formed Michael adduct but also several additional adducts derived from initial Schiff base formation at the C1 carbonyl group of HNE (9). The antigen corresponding to the antibody HNE-pyrrole (BSA modified by 4-oxononanal) was prepared as described previously (12). Absorbed and unabsorbed anti-

bodies were applied to immunoblots of mouse sciatic nerve as well as to adjacent sections of nerves and stained as described earlier.

**Superoxide Dismutase Transgenic Mice.**—Sciatic nerves were dissected from amyotrophic lateral sclerosis-linked superoxide dismutase-1 mutant G85R mice ( $n = 4$ ), end-stage mice ( $n = 2$ ), and age-matched control mice ( $n = 3$ ) (20), and Triton-insoluble fractions were prepared as described above. Equal protein loads were run on SDS-PAGE, electrotransferred to Immobilon, and immunoblotted with antisera to HNE-Michael, HNE-pyrrole, HNE-lysine-lysine, or monoclonal antibody SMI-34. Blots were quantitated as described below, and Student's *t* test was used to compare the values from the different groups.

**Data Analysis.**—All immunoblots were processed following the same time and dilution parameters to allow direct comparisons between various samples. Blots from all samples were stained concurrently for each antibody and for exactly the same time of development. The intensity of immunostained NFH bands was quantitated as reflectivity with an optically enhanced densitometry scanner (QS30; pdi, Inc.). Statistical analysis was performed with analysis of variance Fisher's *post hoc* protected least significant difference test. Comparison of the proximal versus medial and distal segments was performed in addition to comparison of the ages for the mean value resulting from all segments from each of the three mice.

To determine that the loading amount used for analysis was in a linear range, various amounts of mouse neurofilament protein were run on SDS-PAGE and immunoblotted with SMI-34. The immunoreactivity was quantitated for each lane and plotted versus loading amount. The trend line had an  $r^2$  value of 0.953, and the amount used for the study was well within the linear range.

**In Vitro HNE Modification and Quantification.**—Sciatic nerve segments were permeabilized with 0.5% Triton X-100 in Tris-buffered saline. Samples of equal protein were treated with 0.5 mM HNE, prepared as described previously (11, 12), for 2 h, and the reaction was stopped by freezing at -20 °C. Some samples were dephosphorylated (4 units alkaline phosphatase type III (Sigma)/200  $\mu$ g protein in 0.1 M Tris, pH 8.0, with 0.01 M phenylmethylsulfonyl fluoride for 16 h at room temperature or with 50% hydrofluoric acid for 16 h at 4 °C before HNE treatment). Samples were separated on PAGE, electrotransferred to Immobilon (Millipore), and probed with antisera to HNE-Michael and monoclonal antibodies against phosphorylated and nonphosphorylated NFH.

Additionally, neurofilament preparations were incubated in different concentrations of HNE in phosphate-buffered saline (0, 31.25, 125, 500, and 2000  $\mu$ M) for 16 h. After rinsing, blots were immunostained with antisera to HNE-Michael and phosphorylated and nonphosphorylated NFH. All blots were developed for the same amount of time. Quantitative analysis was performed by measuring the intensity of immunostained NFH bands by using digital scanning and KS300 software (Zeiss).

## RESULTS

As we found in samples of the brain and peripheral nerve of human, rat, and rabbit from infancy to senility,<sup>3</sup> antibodies to both partly reversible and stable protein HNE adducts show strong recognition of axons in mice (Fig. 1, A and D) from infancy to senility. The reaction is specific for HNE adducts because axonal recognition is blocked by the respective antigen or competing small-molecule ligand (Fig. 1, B and E). Further attesting to the specificity of the antibodies to endogenous HNE adducts, we found that specific absorption of the HNE-Michael antibody with the HNE-lysine product mixture (Fig. 1B), but not HNE adducts of histidine or cysteine (data not shown), blocked immunorecognition, consistent with the positive detection of immunoreactivity with antibodies recognizing the HNE-pyrrole (Fig. 1D) and HNE-lysine-lysine (data not shown) (both exclusively lysine modifications). Thus, the dominant HNE adducts found in axons are on lysines.

In immunoblots of axonal proteins,  $M_r$  200,000 and  $M_r$  150,000 bands were the major proteins that displayed HNE adducts (Fig. 2), as was found previously for brain tissue.<sup>2</sup> These bands were determined to be NFH and NFM by using specific antibodies

<sup>3</sup> M. A. Smith, L. I. Szewda, L. M. Sayre, and G. Perry, unpublished observations.

FIG. 1. Axons of the sciatic nerve are strongly recognized by antibodies to HNE-Michael (A) and blocked by immunoadsorption with HNE-lysine (B) but not HNE-cysteine or HNE-histidine (data not shown). These axons show prominent phosphorylated NFH (SMI-34) (C). Adjacent serial sections are shown. Axons also show prominent HNE-pyrrole immunoreactivity (D) that is blocked by the antigen (E) and corresponds to the location of phosphorylated NFH (SMI-34) (F). Adjacent serial sections are shown. Scale bar, 25  $\mu$ m.

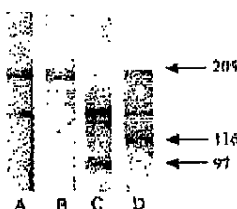
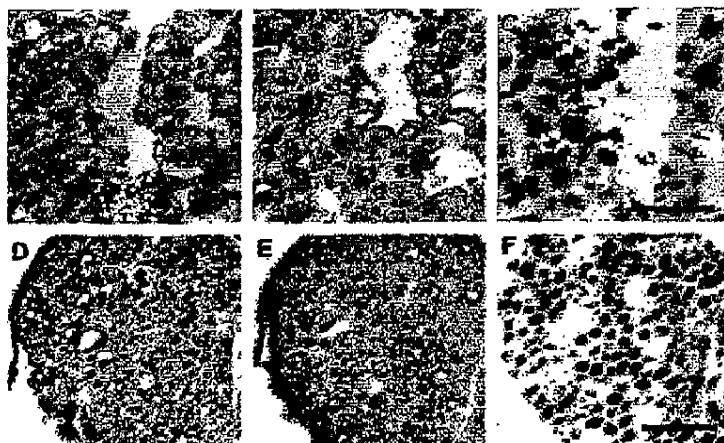


FIG. 2. HNE adducts in axons, defined by HNE-Michael antibody recognition, are primarily associated with NFH and NFM (A) as it is for HNE-pyrrole and HNE-lysine-lysine (data not shown). The high molecular weight bands co-align with NFH as defined by SMI-34 (B), whereas the lower band corresponds with NFM as defined by anti-NFM (C). Coomassie Blue staining of the enriched neurofilament fraction present on the immunoblot is also indicated (D). Molecular weight markers are indicated in thousands.

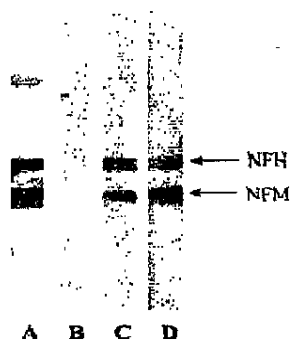
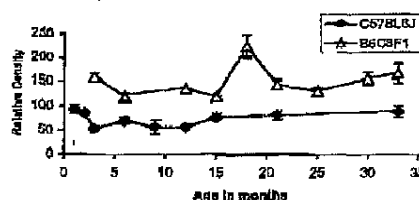


FIG. 3. The Michael adducts formed by HNE are limited to lysine adducts because the antibody to HNE-Michael (D) that recognizes both NFH and NFM on an immunoblot of mouse sciatic nerve is absorbed by HNE-lysine (B) but not by HNE-cysteine (A) or HNE-histidine (C).

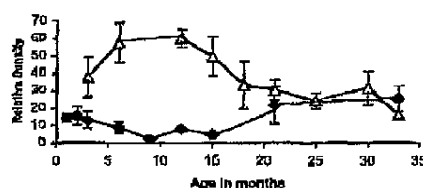
(Fig. 2). Selective absorption of HNE-Michael antibody showed that only HNE-lysine, and not HNE-histidine or HNE-cysteine (Fig. 3), blocked staining, providing additional evidence that modification primarily involves lysine residues.

The molecular weights and high lysine content of NFH and NFM make them candidate targets of HNE adduction. Comparison of the protein bands from mouse sciatic nerve recognized by the antibody to phosphorylated NFH and NFM and the HNE-Michael, HNE-pyrrole, and HNE-lysine-lysine antibodies showed co-alignment (Fig. 2). Note that both NFH and NFM contain similar sequence motifs, explaining the shared

#### A Phosphorylated NFH



#### B HNE-Michael



#### C HNE-Pyrrole

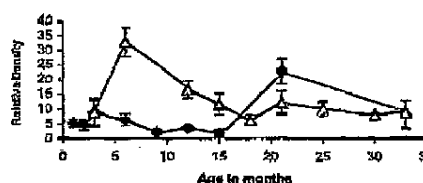


FIG. 4. Comparison of immunoreactivity of NFH on immunoblots containing equal protein loads of sciatic nerve protein with monoclonal antibody to phosphorylated NFH (SMI-34) (A), antisera to HNE-Michael (B), and HNE-pyrrole (C) in two strains of mice. Similar data were obtained for HNE-lysine-lysine (data not shown). Three mice from each age group for each strain were examined, and the data shown are the average of all animals, where the value for each animal is the average of the medial, proximal, and distal segments (i.e. nine samples were analyzed for each point). Graphs show the relative reflective density of immunoreactive bands at  $M_r$  200,000. Error bars,  $\pm$ S.E.  $n = 3$ .

epitopes (21), and, pertinent to the discussion here, have a high lysine content. NFH and NFM not only have a high lysine content but also turn over very slowly in the axon, both properties that should make them susceptible to protracted oxidative attack during aging. Furthermore, NFH and NFM proteins in the distal axon are generally older than those in the proxi-

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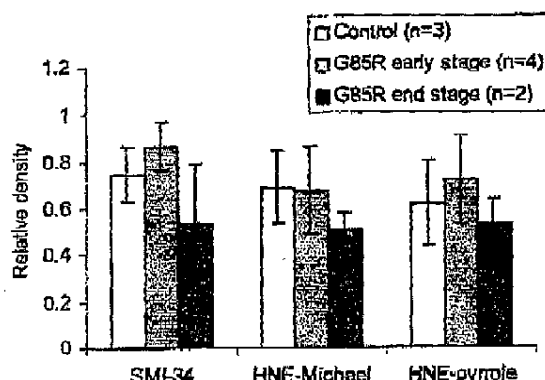


Fig. 5. No significant differences are noted for the recognition of NFH by antibodies to HNE-Michael or HNE-pyrrole when axons of transgenic mice overexpressing mutant human superoxide dismutase-1 (G85R), early or end stage, were compared with control mice. Error bars,  $\pm$ S.E.

mal segments, and this might explain why we noted greater HNE adducts for axons in white matter *versus* cell bodies. Therefore, by comparing proximal and distal segments from mice of various ages, we assessed whether adduction of HNE to high molecular weight neurofilament subunits reflected its long residence time, focusing on NFH because it exhibits a higher state of HNE adduction, consistent with its higher lysine content (21).

We compared HNE-Michael (Fig. 4B), HNE-pyrrole (Fig. 4C), and HNE-lysine-lysine (data not shown) adduction products on NFH (Fig. 4A) and found no significant differences in the extent of adduction for distal, medial, or proximal segments for any of the ages analyzed; therefore, the data for all three segments were averaged. Mice of various ages (1–33 months) were analyzed. Statistical analysis was performed comparing all data points. Although no significant trends were found when comparing overall aging, some significant differences were found when comparing individual time points. Notably, HNE-Michael showed a slight but statistically significant increase with aging from 15 months to greater ages in B6C3F1 mice, whereas with C57BL6J mice, there was a significant decrease over the same period (Fig. 4B). With the stable HNE-pyrrole, B6C3F1 mice did not show a statistically significant increase with aging but did so over the age period of 6–12 months, although the levels in the C57BL6J mice generally remained constant (Fig. 4C) over all the age ranges evaluated. Furthermore, total levels of NFH showed no statistical trend of change over aging, marked by phosphorylated NFH (SMI-34; Fig. 4A) and nonphosphorylated NFH (SMI-32; data not shown). It is particularly important for the HNE-pyrrole modification that levels of NFH or HNE adducts on NFH do not generally increase on aging because it is considered chemically irreversible.

We also considered whether levels of HNE adduction to NFH might be altered under conditions of oxidative stress. Therefore, we examined levels of HNE adducts on NFH in sciatic nerves from transgenic mice overexpressing the human superoxide dismutase-1 G85R mutation. These mice are a model of oxidative stress (22). No significant increases or decreases were noted for the recognition of NFH by antisera to HNE-Michael or HNE-pyrrole when comparing G85 mutants ( $n = 4$ ) and end-stage ( $n = 2$ ) with age-matched controls ( $n = 3$ ) (Fig. 5).

We next considered whether the *in vitro* modification of NFH was at a maximum. *In vitro* treatment of intact permeabilized sciatic nerve or enriched neurofilament fractions with HNE showed that NFH and NFM are both susceptible to additional

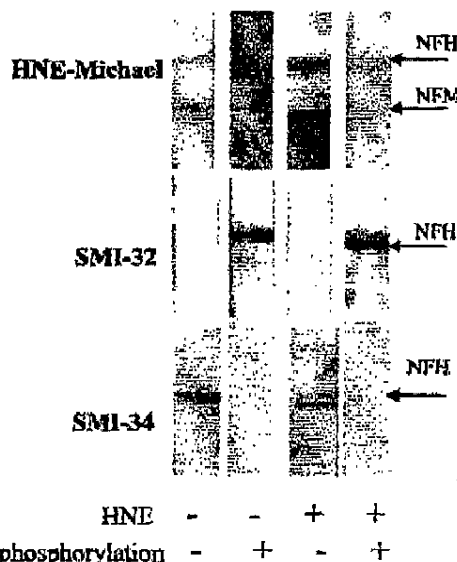


Fig. 6. *In vitro* treatment of permeabilized sciatic nerve with HNE results in adduction levels on NFH and NFM above basal levels as recognized by antisera to HNE-Michael (HNE-Michael) and HNE-pyrrole (data not shown). Phosphates are required for the reaction of HNE with NFH and NFM because dephosphorylation of the axonal samples prior to HNE blocked additional adduction. Antisera for nonphosphorylated NFH (SMI-32) and phosphorylated NFH (SMI-34) are shown to indicate NFH phosphorylated state.

adduction levels over those found *in vivo*. As *in vivo*, the *in vitro* adduction is specific for NFH and NFM, although there are other cytoskeletal proteins such as actin, tubulin, and the low molecular weight neurofilament protein (NFL) present. This specificity of adduction for NFH and NFM is related not only to the high lysine content of both subunits but also to their high level of phosphorylation because dephosphorylation of the proteins before HNE treatment rendered them refractory to HNE adduction *in vitro* (Fig. 6).

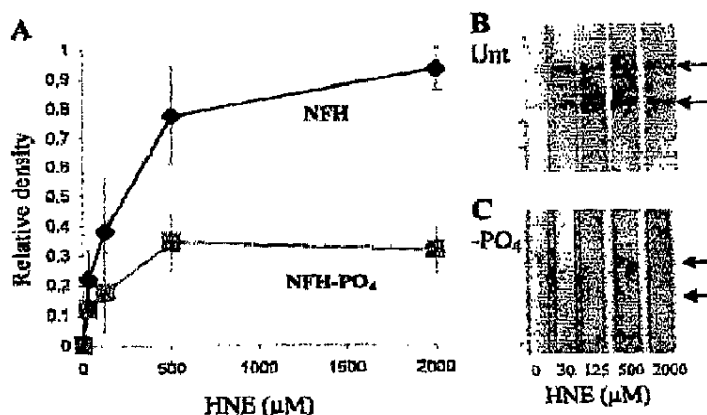
Incubation of enriched mouse neurofilament protein ( $n = 3$  mice) with increasing concentrations of HNE showed that HNE immunoreactivity of NFH significantly increased ( $r = 0.825$ ;  $p = 0.0023$ ). In contrast, prior dephosphorylation of NFH made NFH resistant to HNE adduction ( $p = 0.064$ ) (Fig. 7).

## DISCUSSION

In this study, we demonstrate, both *in vivo* and *in vitro*, that NFH is a selective target of HNE attack and the major protein displaying HNE adducts in the sciatic nerve, as shown here, and in the brain.<sup>2</sup> This finding is not surprising, considering the multiple lysine repeats in NFH and, to a lesser extent, NFM and the high susceptibility of lysine to adduct formation. The additional finding that NFH adduction is controlled by phosphorylation state not only correlates with the selectivity but is also consistent with the high level of HNE adducts in white matter and peripheral nerve, both loci for a high level of NFH phosphorylation. A finding of note is the relatively constant levels of HNE adduction found throughout life and along the entire length of the axon, suggesting a regulated mechanism. If there were not a mechanism to remove NFH modified by HNE, we should note striking increases with aging as well as in distal segments and under conditions of oxidative stress. Candidates for maintaining HNE-NFH dynamics are proteolytic removal of the adducts and competitive trapping of HNE in reversibly formed adducts by glutathione or some unknown mechanism. The fact that similar patterns of adduct formation

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Fig. 7. Incubation of neurofilament fractions with increasing concentrations of HNE followed by immunoblotting demonstrates increasing HNE-Michael formation when neurofilaments have normal phosphorylation levels (NFH) (A and B), whereas, in contrast, there is little reactivity if the phosphates have been reduced by prior alkaline phosphatase treatment (NFH-PO<sub>4</sub>) (A and C).  $n = 3$ .



over the lifetime of mice were seen for reversible and two irreversible modifications of HNE and that HNE adduct formation was not saturated *in vivo* either suggests that highly adducted species of NFH may be removed by proteolysis or reflect the steady-state concentration of HNE in the cytoplasm. In preliminary studies, we found that HNE-modified NFH levels were not altered by treating exposed sciatic nerve to the protease inhibitor leupeptin supplied with hypertonic infusion, but we do not know whether leupeptin is the appropriate inhibitor for the putative proteolytic activity or whether the infusion was an effective means of delivery. Clearly, more definitive approaches are necessary to resolve the mechanisms controlling HNE adduct levels.

One possible implication of HNE adduction on NFH and NFM may be a role for neurofilaments in augmenting the scavenging of aldehydes by neurons, thus protecting other proteins from damage. This function may be especially important in long axons, where protein turnover is low. Here, NFH may act to sequester aldehydes, protecting critical active sites on proteins from oxidative attack. Such a mechanism may be required in the face of the high metabolic activity of the axon that has numerous mitochondria traversing it and likely generating significant amounts of lipoxidation products. The possibility that NFH and NFM may function in part as aldehyde scavengers is further supported by animal and human intoxication studies that indicate selective involvement of neurofilament abnormalities (23).

The observation that NFH and NFM susceptibility to HNE was reduced by dephosphorylation not only suggests that conformation and exposure brought about by phosphorylation play a key role in NFH and NFM susceptibility to modification but also suggest that NFH and NFM adduct formation is associated with phosphorylation upon entry into the axon. The observation that HNE adducts increase in cell bodies in AD (6, 7) is consistent with increased NFH and NFM phosphorylation in the cell body in disease states (24). Similarly, phosphorylated NFH and NFM accumulate in the cell body in amyotrophic lateral sclerosis (25), a condition linked in some cases to mutations in superoxide dismutase-1, presumably associated with increased oxidative stress. Consistent with the scavenger hypothesis, overexpression of NFH and NFM in mice also overexpressing mutated superoxide dismutase-1 delays the onset of loss of motor function and death (26). Intriguingly, we found no increase in HNE adduct levels on NFH in our analysis of singular superoxide dismutase-1 mutant mice. NFH and NFM are proteins unique to vertebrate axons and serve, in part, to determine axonal caliber. The coupled facts that NFH-HNE and NFM-HNE are observed in young animals and that the major target of carbonyl compounds like HNE in both brain

and peripheral nerve is NFH and NFM *in vivo* as well as *in vitro* suggest that the physiological role of NFH and NFM is consistent with an ability to protect the axon from toxic products of oxidative stress throughout life. Certainly, the implications raised here require further study to demonstrate the protective function of NFH and NFM scavenging of carbonyl compounds as well as the biochemical mechanism physiologically maintaining constant HNE-NFH and HNE-NFM levels throughout life.

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